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14. ABSTRACT

The research supported by the Department of Defense Breast Cancer Research Program (BCRP) studies whether the polyphosphate survival function is conserved between prokaryotes and eukaryotes and specifically whether polyphosphates are involved in the increased survival of breast cancer cells. While in the process of developing modified MCF-7 breast cancer cells, an exciting opportunity arose to investigate polyphosphates and their effect on DNA damage response. It is well documented that DNA damaging agents can cause genomic instability and lead to various forms of cancer including breast cancer. Without proper cell cycle checkpoints that trigger repair of the damage or induction of apoptosis, genetic mutations can be propagated, possibly initiating tumorigenesis. Functional analysis of DNA damage response, cell cycle checkpoints which involve BRCA1, genome integrity, and tumor evolution will build the knowledge of the mechanisms involved in breast cancer. The principle investigator ceased this opportunity to study polyphosphates and DNA damage response with respect to cell survival and DNA repair mutagenesis and the DNA damage response (SOS response) which follows extensive DNA damage. It has been discovered that: Loss of polyphosphates increase the cells ability to survive UV radiation; The decrease in survival is not limited to DNA damage caused by UV; The decrease in survival is not specific to the loss of a specific DNA repair mechanism; Polyphosphate levels transiently increase after UV irradiation, in a time frame consistent with error-prone DNA repair; Polyphosphates influence cell survival in pathways separate from RecA and Pol V; and Pol IV functionality in cell survival requires polyphosphates to directly or indirectly instigate the Pol IV pathway.

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INTRODUCTION

Cellular polyphosphates are long linear polymers of orthophosphate linked by high-energy phosphoanhydride bonds and are ubiquitous, having been found in every organism and tissue examined. These anionic polymers are very dynamic, being continuously synthesized and degraded. They are found in chains with lengths of tens to hundreds of phosphate residues. Numerous functions have been proposed based on their physical properties. These include: ATP substitute and energy source, reservoir of orthophosphates, chelator of metal ions, buffer against alkali, channel for DNA entry, and regulator of stress and survival with prokaryotic model systems (Schroder, 1999). While little information exists about the function of these polymers, emerging studies indicate that polyphosphates promotes cell survival by participating in stress response pathways, with reduced polyphosphate levels compromising how cells respond to and survive environmental stresses. The research supported by the Department of Defense Breast Cancer Research Program (BCRP) of the Office of the Congressionally Directed Medical Research Programs (CDMRP) studies whether the polyphosphate survival function is conserved between prokaryotes and eukaryotes and specifically whether polyphosphates are involved in the increased survival of breast cancer cells.

BODY

During the second year of the predoctoral training grant W81XWH-04-1-0379, the exploration for the role of cellular polyphosphates continued. The role must be significant in basic cellular functions since these high energy molecules are ubiquitously present in every prokaryote and eukaryote examined. It is easy to argue that if polyphosphates did not have a vital cellular function, selective pressures would have prevented their conservation throughout evolution. Over the past year in the field of polyphosphate research, the following numbers of articles were published.

- Enzymatic studies of polyphosphate metabolic proteins (8 articles)
- Genomic modifications to polyphosphate operons (3 articles)
- Laboratory techniques utilizing polyphosphate (9 articles)
- Functional roles for polyphosphate (10 articles)
- Polyphosphate accumulations in microbes (9 articles)
- Bioremediation – waste water treatment (18 articles)

These 57 articles were an increase compared to the ~25 articles in the prior year. Additionally, the number of articles involving eukaryotic complex organisms increases yearly. For example, one article identified polyphosphates' role in human blood coagulation. The future research opportunities of polyphosphates and their relevance to medical conditions is encouraging.

While in the process of developing stable transfections of (PPX1) from *S. cerevisae* into MCF-7 breast cancer cells, an exciting opportunity arose to investigate polyphosphates and their effect on DNA damage response. It is well documented that DNA damaging agents can cause genomic instability and lead to various forms of cancer including breast cancer. Without proper cell cycle checkpoints that trigger repair of the damage or induction of apoptosis, genetic mutations can be propagated, possibly initiating tumorigenesis. Functional analysis of DNA

damage response, cell cycle checkpoints which involve BRCA1, genome integrity, and tumor evolution will build the knowledge of the mechanisms involved in breast cancer (Deng, 2006)

The principle investigator modified the aims of this study to cease this opportunity to study polyphosphates and DNA damage response with respect to cell survival and DNA repair mutagenesis. A simplified model system was used to study this relationship. Prokaryotes have much simpler cell structure and are the root of the evolutionary development of higher organisms. By first understanding the functionality in this model system, the future research of polyphosphates in breast cancer cells can be more directed and more efficient. Therefore, *Escherchia coli* have been used to study polyphosphates effect on DNA damage and the DNA damage response (SOS response) which follows extensive DNA damage.

The SOS response activated by DNA damage is the coordinated expression of genes that are normally repressed by LexA. Derepression of this system leads to the syntesis of several proteins that are involved in either DNA repair or recombination with the overall goal to secure genomic stability (Wagner *et al.*, 1999). The system stimulates transcription of over 20genes and operons, including genes producing DNA polymerases (Pol II, Pol IV and Pol V) (Walker, 1996). While majority of the genes are unique to the SOS response, some of these genes also have been identified to be activated in general stress response pathways.

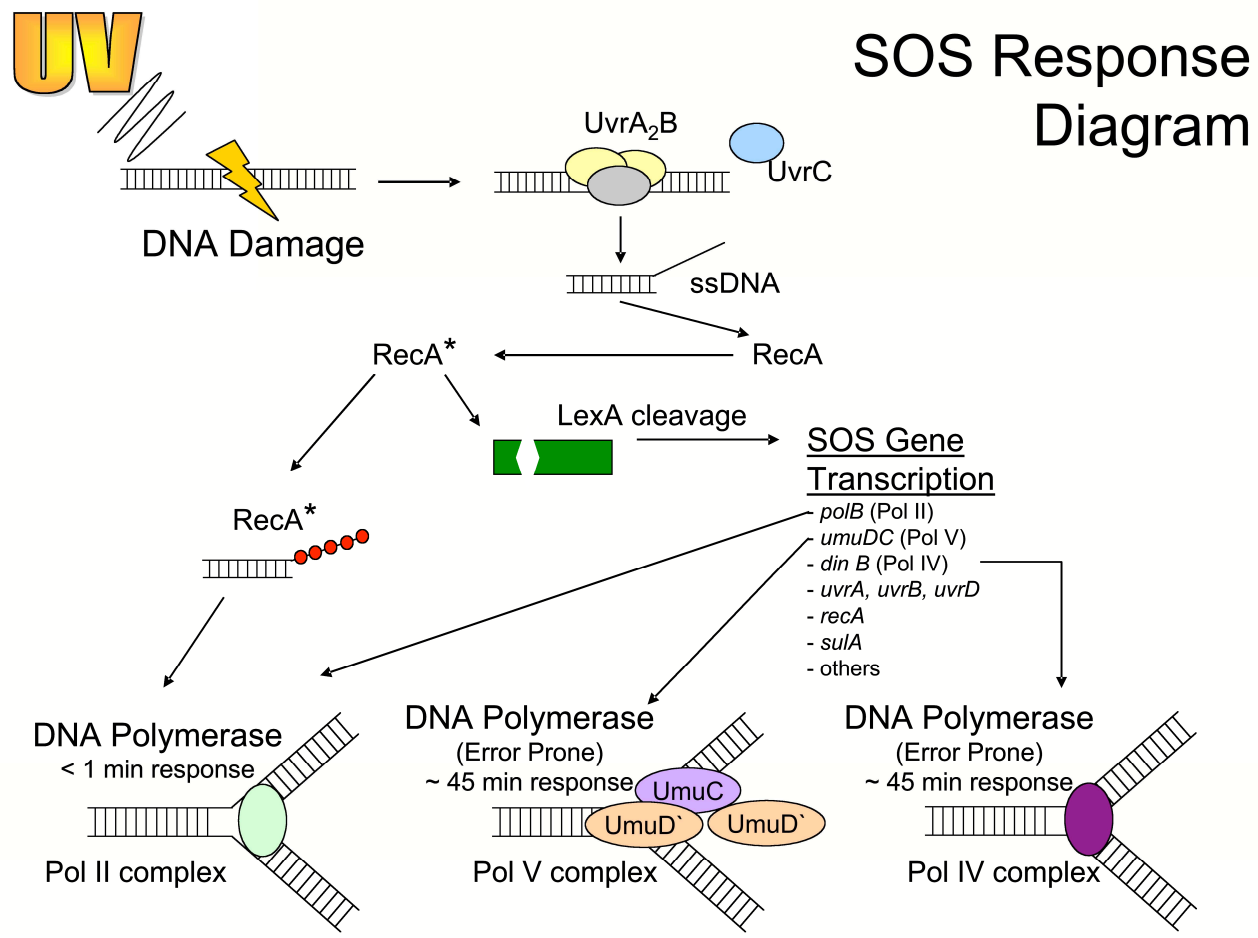

Figure 1: DNA Damage Response Diagram for *E. coli*

Current evidence that links polyphosphates and the SOS response involves *recA* and *umuDC*, which code for two SOS-related proteins. Normally these genes have increased

expression during the SOS response. However, cells with reduced quantities of polyphosphates do fail to have the same increased level of *recA* and *umuDC* expression after significant DNA damage (Tsutsumi *et al.*, 2000). Recently it was suggested that polyphosphates directly or indirectly regulate DNA polymerase activity or fidelity of Pol IV during adaptive mutagenesis (Stumpf and Foster, 2005). To expand the knowledge of polyphosphates' relationship to DNA damage response, this research examines the mechanism by which polyphosphates participate in cell survival after DNA damage. Below are the current results linking polyphosphates to genomic stability following DNA damage.

Results

Polyphosphates increase cell viability after UV irradiation

PPK, the dominant polyphosphate biosynthetic protein, is expressed from the *ppk* operon in the *E. coli* genome. Disruption of this operon to prevent synthesis of polyphosphates results in a greater than ten-fold lower concentration of cellular polyphosphates. Following exposure to environmental stress, polyphosphate levels in wild type increase significantly, while the levels in PPK knockout cells remain at the low basal level.

E. coli cells lacking PPK have decreased survival after exposure to UV radiation. Wild type and PPK^{-} cells were grown, treated and irradiated by UV light. Under all conditions, PPK^{-} cells had decreased survival for exposures ranging from 0 to 150 J/m^2. (Figure 2) During preliminary experiments it was witnessed that variances in initial conditions, such as cell density, led to significant fluctuations in the results: while each experiment showed increased sensitivity for the PPK^{-} cells, the degree of survival after exposure to 150 J/m^2 could range from a 10 fold to 100 fold difference relative to wild type cells depending on initial culture conditions. Therefore, the radiation procedure was performed consistently to ensure the same exposures across all of the samples and experiments. Regardless of the growth phase of the cells being examined, exactly 5.0 x 10^9 cells in a constant volume (20 mls) were exposed to 254 nm UV light in a 100 mm petri dish to assure uniform exposure of the cells from experiment to experiment.

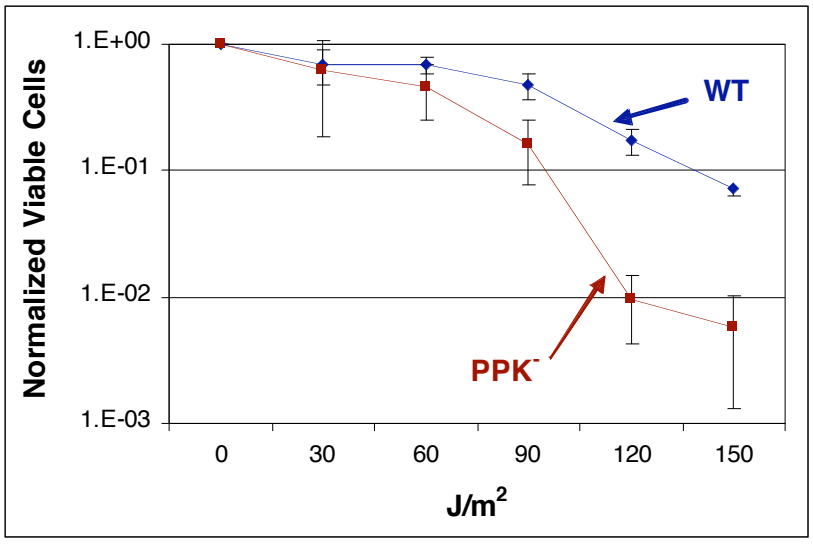

Figure 2: Cells without PPK (PPK^{-}) have greater sensitivity than wild type cells (WT) after UV exposure.

To confirm that the increased sensitivity resulted from the lack of PPK, the PPK⁻ cells were transformed with a plasmid (pPPK) carrying the *ppk* gene behind an inducible BAD promoter (Guzman *et al.*, 1995). These cells were rescued and behaved like wild type cells, while cells transformed with the vector alone (pBAD) had no effect (Figure 3). Measurement of polyphosphate levels showed a 500 fold increase in polyphosphate concentration in PPK⁻ /pPPK cells, verifying the overproduction of PPK and heightened synthesis of cellular polyphosphates. The level of polyphosphates remained unchanged in PPK⁻ cells transformed with pBAD (Table 1).

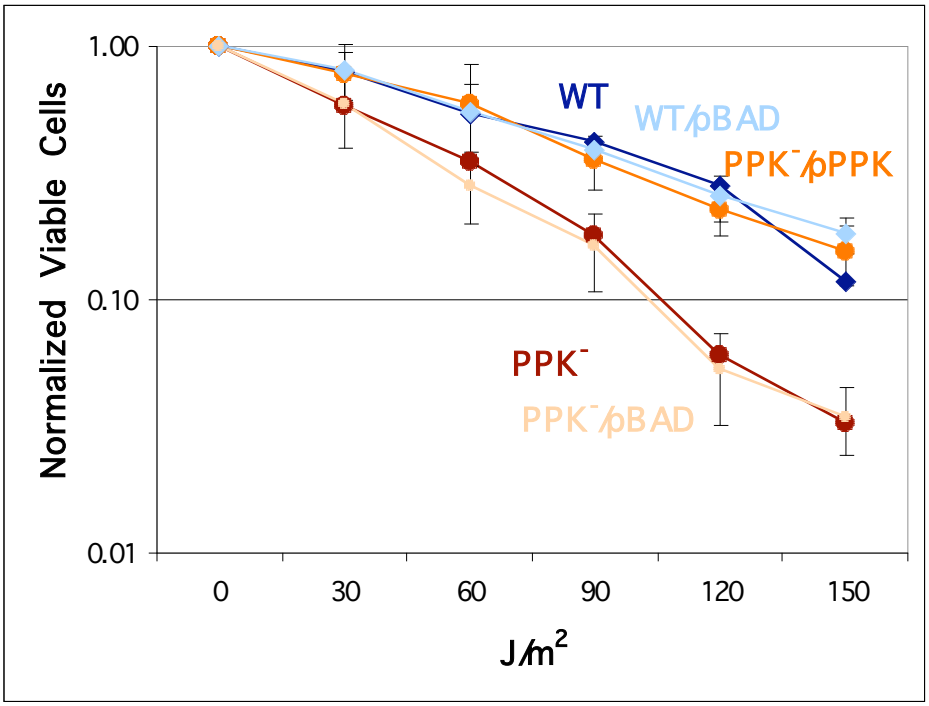

Figure 3: Cells without PPK (PPK⁻) can be rescued by a plasmid over expressing *ppk*.

TABLE 1. Polyphosphate concentrations (pmol/mg protein) after UV irradiation for cultures in stationary phase

		TIME AFTER EXPOSURE
STRAIN	**BEFORE EXPOSURE**	**60 (min)**
Wild type (MG1655)	300	1,498
PPK⁻ (BT2003A)	30	81
BT2003A/pPPK	11,150	61,393
BT2003A/pBAD	43	151

Whether in exponential or stationary growth phase, the wild type cells have increased viability compare to the PPK⁻ cells following UV exposure (150 J/m^2) (Figure 4). This data suggests that the increased survival observed with DNA damage is not limited only to polyphosphate's involvement in the general stress response that occurs as cells enter the quiescent state as growth conditions becoming limiting. If the difference in survival between wild type and PPK⁻ cells after UV exposure was only seen in stationary phase, it could be argued that polyphosphates are not involved in the SOS DNA repair response and that polyphosphate's involvement in the general/stationary stress response was the sole cause of the difference. However, based upon the data (Figure 4), this is not the case and polyphosphates are involved with the SOS DNA repair response to increase cell survival after UV DNA damage.

The survival phenotype difference between wild type and PPK⁻ cells is not dependent on the culture growth rate. PPK⁻ cells are highly sensitive to UV irradiation during slow exponential growth when the cultured in a minimal medium (Figure 3 and 4). But the effect of polyphosphates on survival is not limited to slow growth. PPK⁻ cells rapidly growing in a rich medium are still sensitive to UV (Figure 5). The overall survival of both wild type and PPK⁻ cells is more sensitive when growing with a short doubling time (21 min) versus a longer (90 min). The doubling time of rapidly growing cells is shorter than the approximate 45 minute needed to elicit the Pol V/Pol IV SOS response, hence causing DNA damage to be propagated before complete repair can occur (Pham *et al.*, 2001). This agrees with previous studies that showed the difference in cell survival based solely on the growth rate (Morton and Haynes, 1969); (Stapleton, 1955); (Stapleton and Engel, 1960).

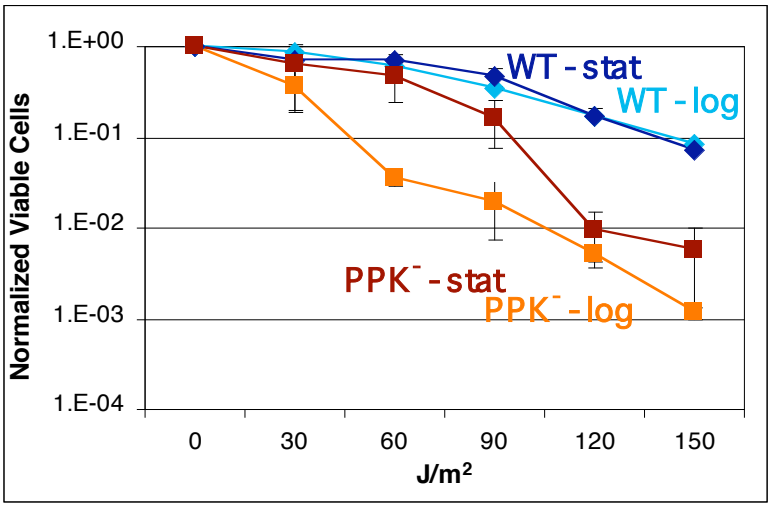

Figure 4: Decreased survival for PPK⁻ cells is independent of growth phase. WT and PPK⁻ cells were grown in slow growth MinA medium and irradiated with UV while in either log or stationary growth phase.

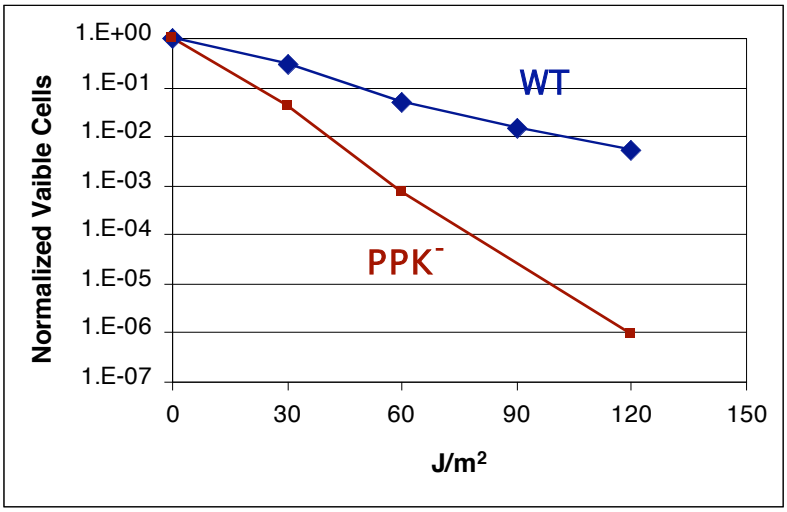

Figure 5: Decreased survival of PPK⁻ cells is independent of growth rate. WT and PPK⁻ cells were grown in rapid growth LB medium and irradiated with UV while in log growth phase.

Polyphosphate levels rise after UV Exposure

Elevations in polyphosphate levels have been observed following exposure to certain environmental challenges that activate the general stress response pathway (Ault-Riche *et al.*,

1998). After exposure to UV, there is a transient increase in polyphosphate concentration in wild type cells (Figure 6). The levels of polyphosphate increased approximately four-fold in stationary phase cells following their exposure to UV. Polyphosphate levels began to increase within 30 min after irradiation and returned to normal levels 1.5-2 hours later (Figure 6). This increase in polyphosphate concentration occurs in the same time frame as the SOS mediated induction of error-prone polymerases Pol IV and Pol V. The PPK⁻ cells did not show any increase in polyphosphates at any time after the UV exposure.

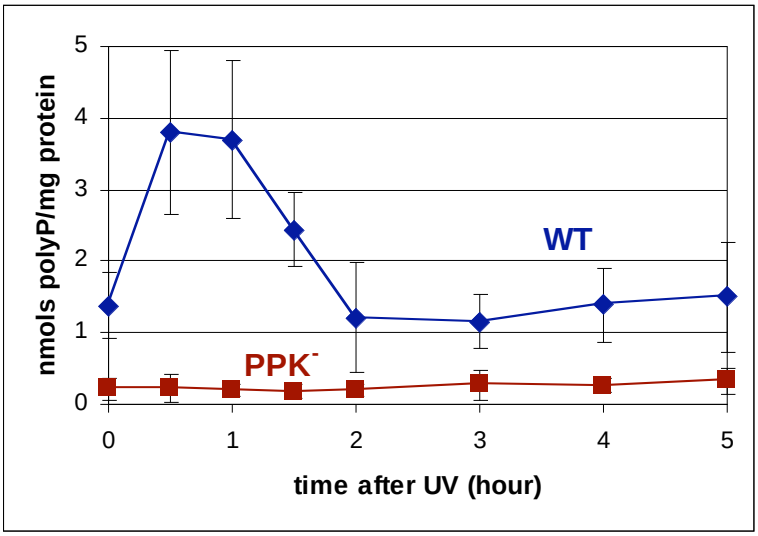

Figure 6: Cellular polyphosphate concentrations following UV irradiation.

PPK Knockout Cells are Sensitive to Other DNA Damaging Agents

To test if the decreased viability was limited to DNA damage caused by UV irradiation, wild type and PPK⁻ cells were cultured in media that contained varying concentrations of cisplatin (1 hour at 37 °C). The chemotherapeutic drug cisplatin forms DNA adducts through intra or inter-strand crosslinks, thus causing DNA damage and inhibiting cellular processes, including replication and transcription (Wozniak and Blasiak, 2002). The PPK⁻ cells have increased sensitivity to cisplatin, similar to that seen with UV exposure (Figure 7). There is nearly a 100-fold difference in survival when the cells were exposed to 50 μg/ml cisplatin. Even though cisplatin forms a different type of DNA lesion, it also triggers the nucleotide excision repair mechanism and the SOS response, like UV irradiation. As expected, polyphosphate deficient cells behave in a similar manner for both DNA damaging agents.

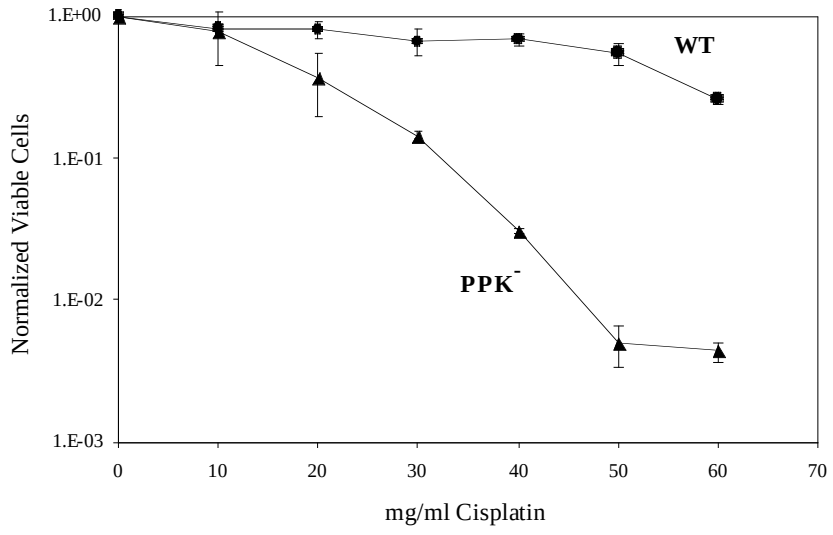

Figure 7: Survival of WT and PPK- cells after exposure to cisplatin.

Gamma irradiation produces double stranded breaks in cellular DNA, thus activating homologous recombination for DNA repair, a repair mechanism different than those for the above DNA damaging agents. Therefore, the viability of wild type and PPK^{-} cells after gamma irradiation was measured to determine if polyphosphates' involvement is specific to a repair mechanism. The PPK^{-} cells had decreased viability compared to wild type cells (Figure 8). A difference of over ten fold in survival appears when the two cell types are exposed to 300 or 400 Gy, suggesting that the role of polyphosphates in cell survival after DNA damage is not limited to a single, specific DNA repair mechanism in the SOS pathway. Instead, it appears to have a more universal role in the SOS response pathway.

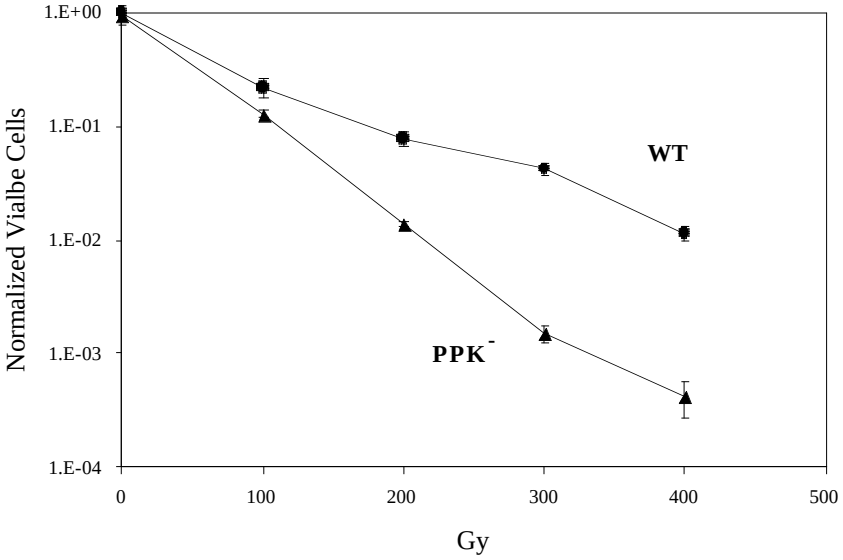

Figure 8: Survival of WT and PPK^{-} cells after exposure to gamma irradiation.

Epistatic Relationships between PPK and known DNA Damage Response Participants

If two proteins are solely on the same pathway, knocking out either one will have the same effect, as will knocking out both. In contrast, if one or both of the proteins are involved in independent pathways, knocking our both will give a different phenotype than the single knockouts of each protein (Figure 1).

Such an epistatic study was carried out between the gene encoding PPK and five genes for proteins known to be involved in the SOS response. UvrA participates in the nucleotide excision repair mechanism. RecA, a core protein in the SOS response, recognizes and binds single stranded DNA that serves as a signal of DNA damage, and thereby induces the SOS response. DNA polymerases II, IV, and V all are induced by the SOS response. While Pol II restarts replication, Pol IV and Pol V are error prone polymerases which are able to replicate pass damaged DNA (Figure 1).

UvrA epistatic studies show UvrA knockout cells are extremely sensitive to UV irradiation and have only 0.000001% survival even after exposure to low levels of UV (Figure 9). This severe sensitivity to UV prevented a difference from being detected between the UvrA^{-} and the PPK^{-}/UvrA^{-} cells.

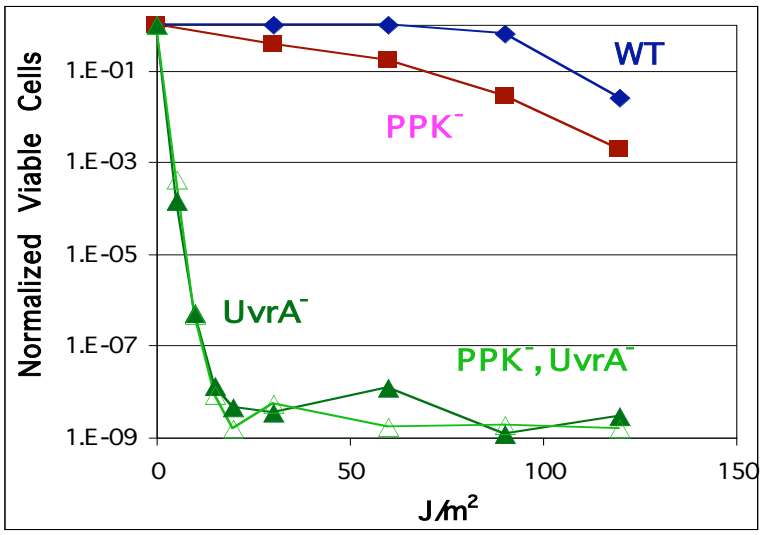

Figure 9: Cellular survival after UV irradiation for UvrA knockouts in wild type and PPK$^-$ cells.

Wild type, single PPK$^-$ or RecA$^-$ knockout, and PPK$^-$/RecA$^-$ double knockout cells were grown to stationary phase, exposed to UV irradiation, and their cell survival measured. The cells which are unable to synthesize polyphosphates are more sensitive to UV irradiation than wild type cells as shown in Figure 2. However, cells that do not have the RecA protein are substantially more sensitive than wild type or PPK$^-$ cells (Figure 10). The double knockout cells appear to be even more sensitive, with an additive increase in sensitivity. This indicates that polyphosphates and RecA may participate in different pathways, with both effecting the cells ability to survive.

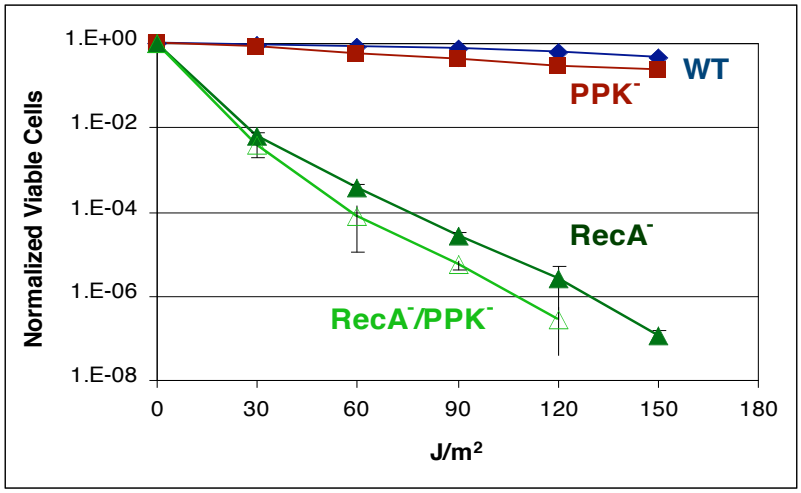

Figure 10: Cellular survival after UV irradiation for RecA knockout in wild type and PPK$^-$ cells

Knockouts of the *polB* gene were previously published to not have increased susceptibility to UV when compared to wild type cells (Bhattacharya and Beck, 2002). Therefore, it was not surprising to find that the cells without Pol II have the same sensitivity to UV as wild type cells and that double knockout cells show the same survival phenotype as PPK$^-$ cells (Figure 11).

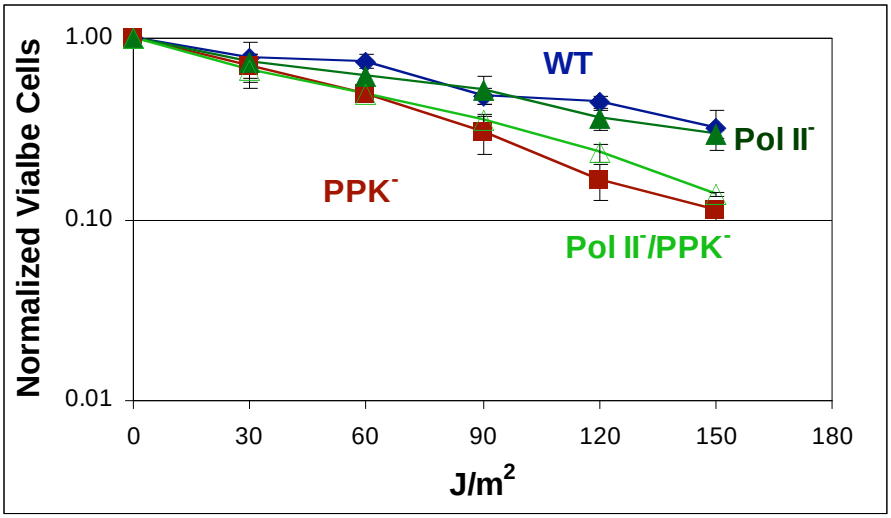

Figure 11: Cellular survival after UV irradiation for Pol II knockout in wild type and PPK⁻ cells

The epistatic analysis of PPK and polymerase V (Pol V) detected potential participation in different pathways for polyphosphates and Pol V (Figure 12). Pol V is an error-prone polymerase, activated by the SOS response, that can replicate past DNA lesions. Pol V is a strong component of the cell survival after severe DNA damage. Wild type, PPK⁻⁻, Pol V⁻⁻ and PPK⁻⁻/Pol V⁻⁻ cells were grown to stationary phase, exposed to UV irradiation and their cell survival measured. Cells that did not have Pol V are more sensitive than wild type or PPK⁻⁻ cells (Figure 12). The double knockout cells appear to be even more sensitive, with an additive increase in sensitivity. This indicates that polyphosphates and Pol V may participate in different pathways, with both effecting the cells ability to survive.

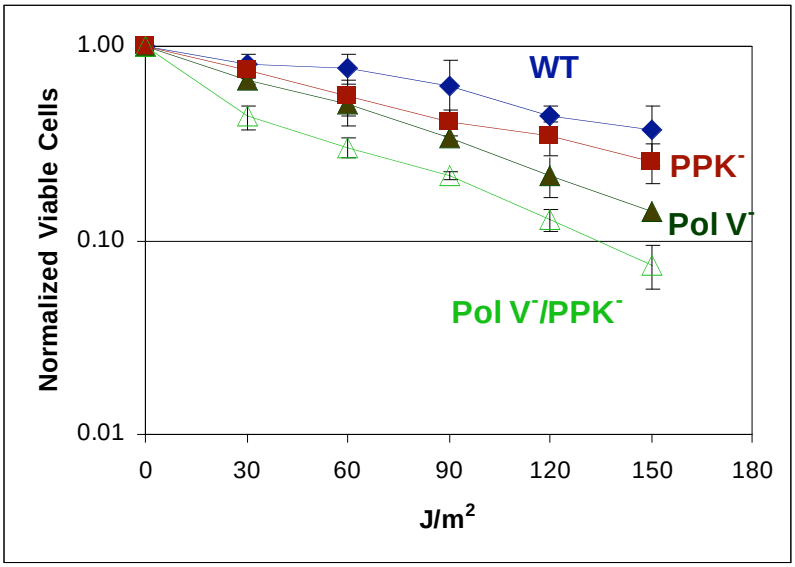

Figure 12: Cellular survival after UV irradiation for Pol V knockout in wild type and PPK⁻ cells

Comparison of survival curves of cells with and without Pol IV and PPK indicates that the double knockout cells have the same survival phenotype as the single PPK knockout cells. This was not expected since Pol IV is another error prone polymerase like Pol V. They both belong to the same protein family and perform similar functions in the SOS response.

Interestingly, the Pol IV single knockout cells have even greater sensitivity than the Pol IV/PPK double knockout cells. This suggests polyphosphates play a role directly or indirectly in Pol IV initiation. Without polyphosphates and/or PPK, the effect on survival caused by the loss of Pol IV is not realized by the cell.

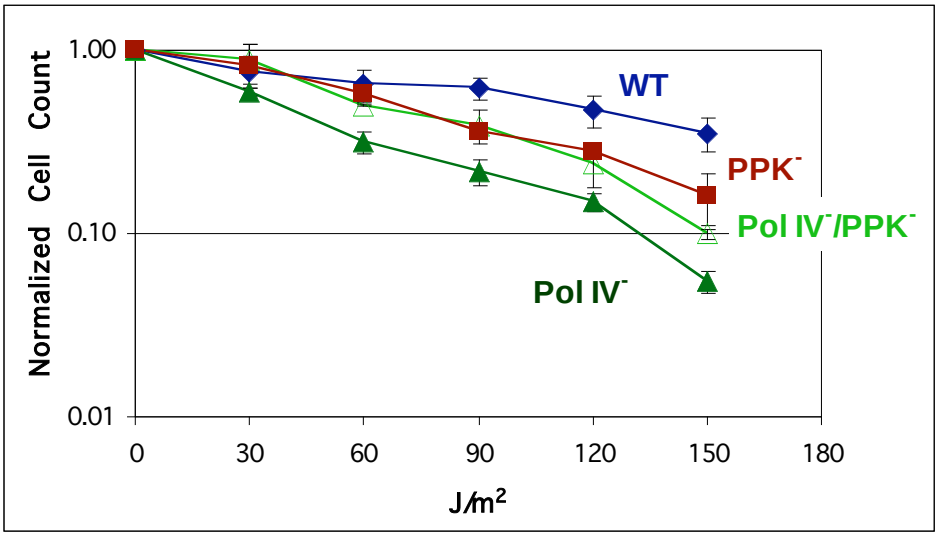

Figure 13: Cellular survival after UV irradiation for Pol IV knockout in wild type and PPK$^-$ cells

Combined, the above research has increased our mechanistic understanding for how polyphosphates participate in cell survival after UV DNA damage. From the epistatic studies, it is clear polyphosphates have an effect on Pol IV activity that is unique to that polymerase. One theory based upon this data is that polyphosphates help initiate the Pol IV mediated repair process. Without polyphosphates, the Pol IV mediated process is not initiated, but other DNA repair mechanisms and error-prone polymerases bestow some cell survival. However, with polyphosphates, but without Pol IV, the Pol IV mediated process can begin but Pol IV does not exist to complete the function, and thus, leaves the cell in an even more vulnerable situation.

It is not known why polyphosphates appear to have an effect on Pol IV and not Pol V even though they belong to the same protein family. The Y family polymerases contains over 50 members with many human forms. Pol κ is the human homolog to Pol IV. A long term goal of this research will be to examine whether polyphosphates also affect Pol κ.

KEY ACCOMPLISHMENTS

Research Accomplishments

- Determined that polyphosphates participates in survival after DNA damage caused by UV radiation, cisplatin exposure and gamma radiation. These three DNA damaging agents form pyrimidine dimers, DNA adducts and double stranded breaks. This suggests that polyphosphates are not the specific to the repair of a specific DNA lesion
- Developed genetically modified strains used for epistatic studies to help determine the molecular mechanism of polyphosphates in DNA-damage survival
- Identified a potential role for polyphosphates in the initiation of Pol IV mediated error-prone DNA repair
- Continued to enhance the procedure to extract polyphosphates from breast cancer cells with excellent and reproducible yield

Training Accomplishments

- Participated in numerous seminars concerning breast cancer associated with the Lombardi Comprehensive Cancer Center
- Presented findings at the ERA OF HOPE, Department of Defense (DOD) Breast Cancer Research Program (BCRP) meeting held in the Pennsylvania Convention Center in Philadelphia, Pennsylvania on June 8-11, 2005
- Participated in weekly seminars and weekly journal clubs that addressed not only research specific to breast cancer but also advances in other cancer research and basic science research. Awareness of other areas of research and new laboratory techniques inspire new ideas for conquering breast cancer.

REPORTABLE OUTCOMES

- ERA OF HOPE, Department of Defense (DOD) Breast Cancer Research Program (BCRP) Meeting – "Effects of Cellular Polyphosphate on Breast Cancer Cell Survival" June 8-11, 2005
- Georgetown University Department of Biochemistry and Molecular Biology Graduate Student Data Presentation – "Role of Cellular Polyphosphates in Enhancing Survival after UV Induced DNA Damage in *Escherichia coli"* October 17, 2005
- Georgetown University Biomedical Research Days Poster Exhibition – "Role of Cellular Polyphosphates in Enhancing Survival after UV Induced DNA Damage in *Escherichia coli"* February 23, 2006
- Construction of strains with genetic modifications to silence following genes: *ppk, recA, uvrA, polB, dinB, umuC*, and double knockouts of *ppk* and each of the other genes
- Developed and confirmed the sensitivity and reproducibility of a procedure to extract and measure polyphosphates in breast cancer cells

CONCLUSIONS

The recipient of this pre-doctoral training grant from the Department of Defense continues to not only build on the knowledge of polyphosphate and breast cancer, but also in her training as a biochemical scientist preparing for a career in breast cancer research. Due to the novel idea of polyphosphates having a potential role in breast cancer, work toward the initial goal of the proposal to examine the physiology of breast cancer cells with altered polyphosphate levels is still ongoing. However, focus during the last year has been on gaining insight into the possible molecular mechanism of polyphosphates in stress survival by exploiting the emerging understanding of how polyphosphates promote survival of prokaryotes following DNA damage. The information gained from the studies described in this report will help direct the continuing examination of polyphosphates in breast cancer cell survival.

As discussed above, key results from the research over the past year are:

- Loss of polyphosphates increase the cells ability to survive UV radiation
- The decrease in survival is not limited to DNA damage caused by UV
- The decrease in survival is not specific to the loss of a specific DNA repair mechanism
- Polyphosphate levels transiently increase after UV irradiation, in a time frame consistent with error-prone DNA repair
- Polyphosphates influence cell survival in pathways separate from RecA and Pol V
- Pol IV functionality in cell survival requires polyphosphates to directly or indirectly instigate the Pol IV pathway

During the third year of the predoctoral training grant W81XWH-04-1-0379, the investigator will continue to research polyphosphates role in cell survival after DNA damage and determine potential homologous functionality in breast cancer cells. The direct or indirect connection discovered between polymerase IV and polyphosphates will identify targets within the breast cancer cells which have potential cell survival effect with polyphosphates.

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